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Anthelmintic Actions of the Cyclic Depsipeptide PF1022A and its Electrophysiological Effects on Muscle Cells of *Ascaris suum*†

Richard J. Martin,[a]* Achim Harder,[b] Michael Londershausen[b] & Peter Jeschke[b]

[a] Department of Preclinical Veterinary Sciences, R.(D.)S.V.S., Summerhall, University of Edinburgh, Edinburgh EH9 1QH, UK
[b] Business Group Animal Health, Institute of Parasitology, Bayer 51368 Leverkusen, Germany

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Abstract: The cyclic depsipeptide PF1022A, given orally to mice, showed very good anthelmintic activity against *Heligmosomoides polygyrus* and *Heterakis spumosa* at 50 mg kg^{-1}. *In vitro*, PF1022A was very active against *Trichinella spiralis* and had good activity against *Nippostrongylus brasiliensis* at 1 $\mu g\ ml^{-1}$. An 18-membered enniatin analogue, JES 1798, showed good activity only against *N. brasiliensis* at 10 $\mu g\ ml^{-1}$. The optical antipode of PF1022A had poor activity even at 100 $\mu g\ ml^{-1}$. The effects of PF1022A on the membrane potential and input conductance of somatic muscle of *Ascaris suum* were examined using a two-microelectrode current-clamp technique. PF1022A did not antagonize the effects of the selective nicotinic agonist levamisole. PF1022A and an analogue, JES 1798, but not the PF1022A antipode, produced a small time-dependent increase in input conductance associated with no potential change. The increase in input conductance did not occur in the Cl^--free bathing solution, suggesting that the increase in input conductance was mediated by Cl^- ions. The addition of high concentrations of Ca^{2+} to the preparation after the addition of PF1022A did not lead to production of Ca^{2+}-activated Cl^- channels, suggesting that its mode of action was not that of a Ca^{2+} ionophore. The mechanism by which the cyclic depsipeptide might increase the Cl^- conductance is discussed.

Key words: cyclic depsipeptide, PF1022A, electrophysiology, anthelmintic, *Ascaris suum*, mode of action, *Heligmosomoides polygyrus*, *Heterakis spumosa*, mouse

1 INTRODUCTION

The 24-membered cyclic depsipeptide PF1022A is a fermentation product of a strain of *Myclia sterilia* that was isolated from microflora on the leaf of the plant *Camellia japonica*, collected from the Ibaragi Prefecture in Japan. The potent antinematodal property of this agent against *Toxocara canis*[1] and *Toxocara cati*,[1] intestinal nematodes of dogs and cats, and against *Ascaridia galli*,[2] an intestinal nematode of chickens, was reported in 1990 and 1992.[1,2] Recently the chemical synthesis of this and the radiolabelled compound have been reported.[3,4]

PF1022A appears to exert its anthelmintic effect on neuromuscular transmission of nematodes rather than by affecting energy metabolism. This is suggested by experiments with PF1022A on the motility of *Angiostrongylus cantonsis*;[5] low concentrations (10^{-13}–10^{-6} g ml^{-1}; 0·1 pM–1 μM) of PF1022A depress the motility of the preparation within 100 min and 50 μM picrotoxin and 30 μM bicuculline and 20 mM Ca^{2+} antagonize the action of PF1022A. These experiments suggest that PF1022A is neuropharmacologically active and that part of the effect of PF1022A may be explained by an antagonism of acetylcholine receptors and/or a GABAergic effect.

* To whom correspondence should be addressed.
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Other novel related cyclic depsipeptides, like JES 1798, a derivative of the naturally occurring enniatins like enniatin A, B, C and beauvercin, which are 18-membered cyclic depsipeptides,[6] have been synthesized.[7]

In this paper experiments on the anthelmintic and electrophysiological effects of PF1022A and JES 1798 are reported, the aim of which was to test further their anthelmintic activities and to determine if PF1022A behaved like a nicotinic antagonist or a GABA agonist.

2 METHODS

2.1 In-vitro experiments

The larvae of the nematode *Trichinella spiralis* were isolated from skeletal muscles and diaphragms of SPF/CFWI mice and stored in sodium chloride solution (9 g litre^{-1}), supplemented with Canesten (20 μg ml^{-1}). Twenty larvae per estimation were incubated in a solution (2 ml) containing: Batcho Capstone, 20; yeast extract 10; glucose 5; KH_2PO_4, 0·8; K_2HPO_4, 0·8 g litre^{-1}; pH 7·2 supplemented with sisomycin (10 μg ml^{-1}) and canesten (1 μg ml^{-1}). The test compound (10 mg) was dissolved in dimethylsulfoxide (DMSO; 0·5 ml) and added to the incubation medium to give final concentrations of 100, 10, 1, 0·1 and 0·01 μg ml^{-1}. The experiment was stopped after 5 days of incubation at 19°C. Activity was assessed on a scale 0–3 where 3 = full activity (all larvae dead); 2 = good activity (<100% but >50% of the larvae dead); 1 = weak activity (>50% of larvae still alive) and 0 = no activity (number of living larvae equals that in the control).[8]

Adult nematodes of *Nippostrongylus brasiliensis* were isolated from the small intestine of female Wistar rats, stored in sodium chloride (9 g litre^{-1}), supplemented with Sisomycin (20 μg ml^{-1}) and Canesten (2 μg ml^{-1}). The incubation of each group of male or female worms was performed in 1·0 ml medium, which was collected for the estimation of the level of activity of the acetylcholinesterase secreted by *N. brasiliensis*. Details of the incubation and determination are as described by Rapson, *et al.*[9] The level of activity of the test compound was assessed on a scale 0–3 where 3 = full activity (95%–100% enzyme inhibition); 2 = good activity (75%–95% inhibition); 1 = weak activity (50–75% inhibition); 0 = negligible activity (<50% inhibition).

2.2 In-vivo experiments

For all experiments, male mice of the strain SPF/CFWI, 16–18 g body weight on receipt, were used. Five animals were housed per macrolon cage and given water and 'Sniff' rat feed, (13-cm feed pellets), *ad libitum*. Mice were given a mixed infection of the tapeworm *Hymenolepis nana* and the nematodes *Heligmosomoides polygyrus*, *Heterakis spumosa* and *Trichinella spiralis*. The infective material from *H. nana* was collected from mouse faeces 14–21 days after infection. Third-stage larvae of *H. polygyrus* were collected from mouse faeces 21 days after infection. *Heterakis* eggs were obtained from female worms isolated from mouse colon 35–42 days after infection and after incubation at 27°C for three weeks. *T. spiralis* larvae were obtained from pepsin-treated skeletal muscles and diaphragm from Wistar rats 20 days after infection.

The mixed infection was produced by an initial oral infection of 90 embryonated *H. spumosa* eggs on day 1; on day 7, 100 *T. spiralis* larvae were given orally; on day 34, 60–70 *H. polygyrus* larvae were given orally; on day 36, 100 eggs of *H. nana* were given orally. Treatment with the test compounds began on day 46 and ended on day 49. On day 57 the mice were killed with carbon dioxide and dissected. *H. nana* were isolated from the small intestine and the number of tapeworms estimated microscopically with a magnification of ×16. *H. spumosa* were isolated from caecum and colon and counted macroscopically. *H. polygyrus* were examined in the duodenum after removal and pressing in a compressor; parasites were counted microscopically with a magnification of ×40. For examination of *T. spiralis* larvae, a 1-cm^2 piece of abdominal muscle was dissected and pressed between two plastic sheets, using a hand press, and the parasites in the pressed muscle examined microscopically (×40). The level of anthelmintic activity against the three species of nematode was graded using the following criteria: 3 = cure (no parasites detectable); 2 = active (<20% of parasites remaining); 1 = trace effect (<50% but >20% of parasites remaining); 0 = ineffective (>50% of parasites remaining). The activity against *H. nana* was graded on a scale 0–3, where 3 = cure (no parasites detectable); 2 = effective (some parasites expelled, tapeworm strobila excreted); 1 = trace effect (tapeworm strobila excreted or macerated) and 0 = ineffective (all parasites remaining).

Test compounds were dissolved or suspended in 'Cremophor' EL (0·2 ml emulsifier g^{-1} test compound) and 0·5 ml of this solution (or suspension) was administered to each mouse daily for four consecutive days. For each test compound, one mouse received the highest dose of 250 mg kg^{-1}. If anthelmintic activity was detected at this dose, lower doses of 100, 50, 25 and 10 mg kg^{-1} were tested down to the level where no anthelmintic activity was detectable.

2.3 Preparation of *Ascaris suum* muscle for electrophysiology

Details of the preparation have been described fully elsewhere[10,11] but they are outlined below. *Ascaris suum* were collected from the local abattoir and maintained in Locke's solution at 35°C and the solution replaced daily. A body flap preparation of *A. suum* was

made from a region about 3 cm from the head and mounted on Sylard in an experimental chamber containing 6 ml of a well-stirred bathing solution maintained at 37°C. The experiments reported here are based on observations on 16 preparations of *A. suum*, each compound (and experiment) was tested on at least two separate preparations.

2.4 Electrophysiological experiments

The preparation was bathed, except where stated, in a low-Ca^{2+} high-Cl^- solution containing sodium chloride, 132; potassium chloride, 2·4; magnesium chloride, 10; glucose, 11; HEPES, 5 mM and DMSO, 10 ml litre^{-1}; pH 7·4, adjusted with sodium hydroxide. The low-Ca^{2+} solution was used to abolish spontaneous depolarizing potentials and high-Cl^- solution was used to enhance Cl^- conductance responses. In some cases a zero-Cl^- solution was used in which all the Cl^- ions were replaced by acetate.

For recording, two glass micropipettes filled with potassium acetate (2 M), were inserted into the bag region of the muscle with the aid of a dissecting microscope and illumination from below. The micropipettes had a resistance of 20–25 MΩ. Constant current-clamp pulses of 40 nA for 1 s were injected at a rate of 0·2 Hz with an Axoclamp 2A to produce hyperpolarizing pulses. The current–voltage relationship of the muscle was examined and found to be linear in the hyperpolarizing ranges used. Current injection and voltage responses were monitored on an oscilloscope and voltage responses recorded on an Electromed two-channel pen recorder. The figures illustrating the electrophysiological responses were prepared directly from the original pen recordings: the width of a trace (envelope of the trace) is inversely proportional to the input conductance. An agar bridge was used as a bath earth. Concentrated stock solutions of the drugs PF1022A, JES 1798 or PF1022A antipode were added in volumes $\lesssim 300$ µl to the well-stirred bath. The stock solutions were made up in the standard bathing solution with DMSO (10 ml litre^{-1}). At this concentration DMSO had no detectable effect on GABA responses. Mixing was complete within 1 min (dye test). The relative molecular masses of PF1022A, PF1022A antipode and JES 1798 are 948, 948 and 556, respectively. The preparation was washed by draining the bath and replacing the bathing solution three times. The significance of effects of PF1022A and PF1022A antipode on input conductance change was compared using values at 10 and 20 min and a two-way analysis of variance with replications.

3 RESULTS AND DISCUSSION

PF1022A had full anthelmintic activity *in vitro* against *T. spiralis* and *N. brasiliensis* at 1 µg ml^{-1} (1·05 µM) (Table 1). Previously we have reported that PF1022A, at similar low concentrations,[3] has full anthelmintic activity *in vitro* against *Heterakis spumosa*, a lumen-dwelling nematode of the mouse. In contrast, the PF1022A optical antipode exerted only a slight anthelmintic activity against *N. brasiliensis* at the high concentration of 100 µg ml^{-1}, whereas the enniatin derivative JES 1798 had good anthelmintic activity *in vitro* at 10-fold higher concentrations against *N. brasiliensis* compared to PF1022A (Table 1).

PF1022A also showed full anthelmintic activity *in vivo* at the oral dose-rate of 50 mg kg^{-1} against *H. polygyrus* as well as against *H. spumosa* in the mouse (Table 2). There was no detectable effect against the cestode *H. nana* or *T. spiralis* larvae in the muscle. We have already reported the activity of JES 1798 *in vivo* against the sheep nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis* at an oral dose-rate of 1–5 mg kg^{-1}, when administered intravenously or orally.[7]

To test the mode of action of PF1022A using electrophysiological methods we chose the nematode *Ascaris suum*. This pig nematode parasite has the advantage of being large enough to allow intracellular recording micropipettes to be used and has been studied extensively using electrophysiological methods. The aim in

TABLE 1

Anthelmintic Activity of PF1022A, PF1022A Antipode and JES 1798 *in vitro* against *Trichinella spiralis* Larvae and *Nippostrongylus brasiliensis* Adults[a]

Concentration (µg ml^{-1})	Trichinella spiralis			Nippostrongylus brasiliensis		
	PF1022A	*PF1022 antipode*	*JES1798*	*PF1022A*	*PF1022 antipode*	*JES1798*
100	3	1	0	3	1	2
10	3	0	0	3	0	0
1	3	0	0	2	0	nd
0·1	2	0	0	0	0	nd

[a] 0 = no activity; 1 = slight activity; 2 = good activity; 3 = full activity. For quantitation of the effect of the test compound see Section 2.1. Each compound was tested in two different experiments ($n = 2$) at each concentration. 1 µg ml^{-1} is equivalent to 1·05 µM PF1022A, 1·05 µM PF1022 antipode or 1·89 µM JES1798.

TABLE 2
Anthelmintic Activity of PF1022A against Different Helminths of the Mouse[a]

Doseage ($mg\ kg^{-1}$)	*Hymenolepis nana*	*Heligmosomoides polygyrus*	*Heterakis spumosa*	*Trichinella spiralis larvae*
100	0	3	3	0
50	0	3	3	0
25	0	2	0	0
10	0	1	0	0
5	0	0	0	0

[a] 0 = no activity; 1 = slight activity; 2 = good activity; 3 = full activity. For quantitation of the effect of the test compound see Section 2.1. Each dose level was tested in two different experiments ($n = 2$) with three mice being used at each dose level.

these experiments was to determine if the anthelmintic effects of the cyclic depsipeptides could be explained by either a nicotinic antagonist action or a GABA agonist action as proposed[5] or both.

Levamisole is a selective nicotinic agonist in *A. suum* and will depolarize the muscle and increase the input conductance.[12] Figure 1 shows that adding 30 μM levamisole to the bath produced a 15-mV depolarization and increased the input conductance of the muscle cell from 2·53 μS to 4·35 μS. Addition of PF1022A to the bath at 10^{-6} g ml^{-1} (1·05 μM) had little effect: after 10 min the input conductance increased slightly to 4·25 μS and there was little effect on membrane potential. The same effect was reproduced in another preparation of *A. suum*. Since the effect of levamisole was not reversed by PF1022A ($n = 2$), it was concluded that PF1022A is not a nicotinic antagonist in *A. suum*.

Piperazine is a regularly used anthelmintic that acts as a GABA agonist in nematodes.[11] Bath-applied piperazine produces a marked dose-dependent increase in Cl^- conductance of the muscle membrane as well as a large hyperpolarizing potential in cells of *A. suum*.[11] We tested the effect of PF1022A as a GABA agonist using the same methods.

Figure 2A illustrates the effect of PF1022A (10^{-6} g ml^{-1}; 1·05 μM) on input conductance in high-Cl^- bath solution and on membrane potential. It can be seen that there is a slow, time-dependent increase in input conductance of the membrane from a resting value of 1·74 μS to 2·00 μS over a period of 30 min. The membrane potential shows no detectable change during this period. The effect of PF1022A was not reversed by washing (not shown). The small time-dependent effect on input conductance was reproducible (Table 3) but it did not occur in the absence of Cl^- in the bath (Fig. 2B), showing that Cl^- ions are involved in mediating the response. Also no conductance effect was observed with the biologically inactive isomer, PF1022A antipode, (Fig. 2C). The change in conductance produced by PF1022A at 10 min and 20 min was significantly greater than that produced by PF1022A antipode ($P < 0{\cdot}001$, F test).

The slow time-dependent effects of PF1022A on conductance and the absence of an effect by the PF1022A antipode suggests that the effect may be related to the anthelmintic action of these drugs. However, it is difficult to explain the small and time-dependent increases in Cl^- conductance produced by the cyclic depsipeptides as a simple GABA agonist effect. First, except for piperazine, the structures of GABA agonists that are active in *A. suum*[13,14] possess a carboxyl group separated from a charged ammonium group by a distance of

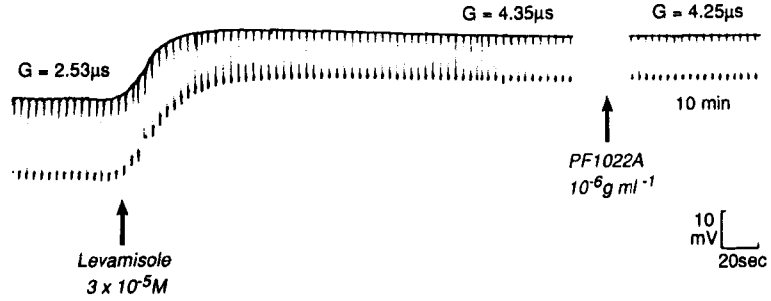

Fig. 1. PF1022A (10^{-6} g ml^{-1}: 1·05 μM) does not antagonise the nicotinic effect of levamisole. Levamisole (30 μM) produces a 15 mV depolarization and an increase in input conductance from 2·53 μS to 4·35 μS but these effects are not antagonized by PF1022A (10^{-6} g ml^{-1}; 1·05 μM). The rapid movement of the pen recorder at the onset and offset of the current injection pulse produces pen skipping but the envelope of the trace (width) is directly related to the input resistance (= 1/input conductance) of the preparation.

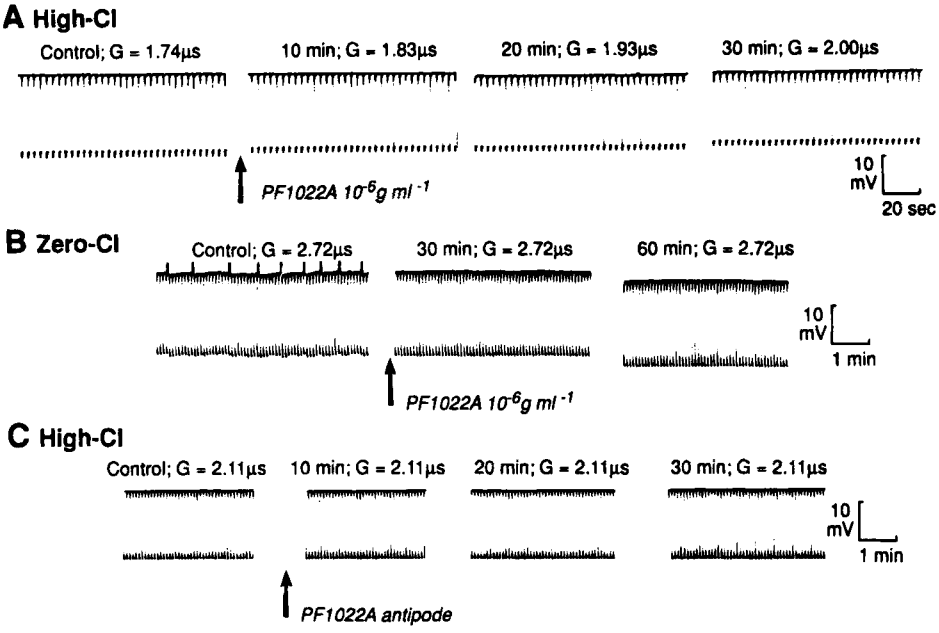

Fig. 2. Effect of PF1022A and PF1022A antipode on the input conductance of the *Ascaris* muscle membrane. Constant current clamp pulses of 40 nA for 1 s were injected at a rate of 0·2 Hz with an Axoclamp 2A to produce hyperpolarizing pulses. A: In high $-Cl^-$ bath solution PF1022A (10^{-6} g ml^{-1}; 1·05 μM) produces a small increase (0·26 μS) in input conductance over a period of 30 min. B: In zero $-Cl^-$ bath solution PF1022A (10^{-6} g ml^{-1}; 1·05 μM) produces no change in input conductance over 60 min. C: In high $-Cl^-$ bath solution PF1022 antipode (10^{-6} g ml^{-1}; 1·05 μM) produces no change in input conductance.

5 Å and, second, GABA agonists produce a marked hyperpolarization and larger increase in Cl^--conductance of the muscle membrane.[10,11] PF1022A does not induce any obvious hyperpolarization.

As a further test of the electrophysiological effects of cyclic depsipeptides, JES 1798, another active anthelmintic analogue of PF1022A, was tested. At 10^{-6} g ml^{-1} (1·89 μM) it also produced a small and slow increase in input conductance of the membrane (Fig. 3). Enniatins like enniatin A, B or C, as well as beauvercin, are known to induce cation fluxes across membranes[7] and might produce a secondary increase in

TABLE 3
Effect of PF1022A, PF1022A Antipode and JES1798 on the Input Conductance of *Ascaris* Muscle and Membrane Potential

	10 min ΔG (μS)	*20 min* ΔG (μS)	*30 min* ΔG (μS)	*10 min* *mV change*
PF1022A (10^{-6} g ml^{-1})				
exp. 1	0·09	0·19	0·26	0
exp. 2	0·18	0·29	0·36	0
exp. 3	0·17	0·17	nd[b]	0
PF1022A antipode (10^{-6} g ml^{-1})				
exp. 1	0·04	0·06	nd	0
exp. 2	−0.05	−0.05	nd	0
exp. 3	0	0	0	0
JES1798 (10^{-6} g ml^{-1})				
exp. 1	0·12	0·26	nd	0
exp. 2	nd	nd	nd	0

[a] The input conductance change following addition of the compounds was determined at 10, 20 and 30 min. The change in membrane potential was determined after 10 min. Each experiment was conducted on a separate preparation made from a fresh *Ascaris*. Two-way analysis of variance with replication showed that the effects of PF1022A on ΔG were significantly ($P < 0·001$, F test) greater than PF1022A antipode.
[b] nd: not determined.

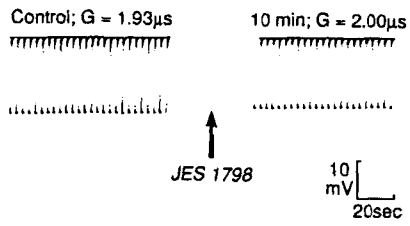

Fig. 3. Effect of enniatin JES 1798 on input conductance of *Ascaris* muscle. In high $-Cl^-$ bath solution JES1798 (10^{-6} g ml^{-1}; 1·89 μM) produces a conductance change, 0·07 μS, after 10 min.

Cl^- conductance, as discussed later. From our observations it can be concluded that cyclic depsipeptides are not potent GABA agonists because (1) they did not produce a marked hyperpolarization and (2) they did not produce a large increase in input conductance.

Ascaris muscles contain Cl^- channels that are activated by an increase in intracellular Ca^{2+} concentrations.[15] The cyclic depsipeptides have a macrocyclic lactone ring structure, so it is possible that they act as an ionophore, carrying cations,[7] including Ca^{2+}, across the cell membrane, leading to the observed increase in membrane conductance. Although experiments were carried out in the absence of added Ca^{2+} ions in the bath, we decided to check for the possibility of PF1022A facilitating the entry of Ca^{2+} ions into the muscle cell leading to activation of the Ca^{2+}-dependent Cl^- channels. Our experiments, however, indicated that the addition of Ca^{2+} ions (2–6 mM) to the bath solution did not enhance the effect of PF1022A on input conductance (not shown). This observation suggests that PF1022A does not produce its effect on input conductance by acting as a Ca^{2+} ionophore.

A number of possible explanations for the mode of action of the active cyclic depsipeptides remain:

1. The cyclic depsipeptides may combine with one of the subunits of the GABA receptor-channel, but not at the GABA binding site, and predispose the channel towards opening; it could then potentiate the action of GABA. We have not looked for GABA potentiation in these experiments. One disadvantage of this explanation is that it predicts toxic effects on the host animal as well as on the parasite; the host animal also has peripheral GABA receptors as well as the parasite.
2. Another explanation is that the cyclic depsipeptides are selectively metabolized by the nematode parasites to a GABA agonist. The anthelmintic action is explained by the local production of a GABA agonist in the parasite but not in the host.
3. The cyclic depsipeptides lead to partial activation of Cl^- channels that are present only in the parasite: the Ca^{2+}-activated Cl^- channels.[15] Such an effect could be produced directly on the channel or by secondary effects, as the cyclic depsipeptides insert into membranes and act as an ionophore leading to changes in intracellular Na^+ or pH. We found no evidence in this study that PF1022A acts as a Ca^{2+} ionophore. The Ca^{2+}-activated channel is, however, also sensitive to intracellular pH changes as well intracellular Ca^{2+} changes.
4. The cyclic depsipeptides have a macrocyclic lactone structure; their effects on muscle tone may be antagonized by pictrotoxin[5] and cyclic depsipeptides have selective toxic effects on nematode parasites. All these observations may be explained if the depsipeptides act in a similar way to the avermectins, also macrocyclic lactones, and lead to opening of glutamate-gated Cl^- channels that are present only in invertebrate species.[16] Recently this receptor has been localized on the *A. suum* pharyngeal muscle, and testing the effects of the macrocyclic lactones on this receptor by electrophysiological means is now possible.[17] These possibilities remain to be tested in future experiments.

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